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Extracts of *Agave americana* inhibit aflatoxin production in *Aspergillus parasiticus*

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Abstract

Toxigenic fungi invade crops prior to harvest as well as during storage and produce harmful, even carcinogenic toxins such as aflatoxins. Since consumers demand safe commodities, and due to enhanced public awareness of the dangers of many synthetic fungicides, the importance of investigating alternative, natural products to control these toxigenic fungi is clear. This study investigated the effect of aqueous extracts of *Agave americana* on growth, conidia and aflatoxin production. *Aspergillus parasiticus* strains SRRC 148, SRRC 143 (Su-1), and *A. parasiticus* SRRC 162, a mutant (*nor*$^-$) that accumulates norsolorinic acid (NOR, an orange-coloured intermediate of the aflatoxin pathway), were first inoculated into Adye and Mateles liquid medium, then plant extracts were added, and incubated at 28 °C for 7 days. Aflatoxin and norsolorinic acid were assayed by HPLC and spectrophotometry, respectively. While the extract of *A. americana* stimulated growth of the studied fungi, conidiogenesis, norsolorinic acid accumulation (in the *nor*$^-$ mutant), and aflatoxin production were significantly affected. The reduction was produced by the extracts at concentrations higher than 5-10 mg/ml, where all types of total aflatoxin analysed (aflatoxins B_1, B_2, G_1 and G_2) were reduced from 64% to >99% in the whole culture, and a reduction of 75% of norsolorinic acid. The results of the present work indicate that extracts of *A. americana* may be promising safe alternatives to harmful fungicides for controlling aflatoxin contamination.

Keywords: aflatoxin biosynthesis, biological control, conidiogenesis, control methods, norsolorinic acid

1. Introduction

Aflatoxins are highly toxic, carcinogenic, and teratogenic polyketide secondary metabolites, produced by many strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, and by only a few strains of *Aspergillus tamari* and *Aspergillus nomius* (Bhatnagar and García, 2001). The biosynthesis of aflatoxin has been studied for a number of years (Bennett and Klich, 2003; Keller *et al.*, 2005; Wen *et al.*, 2005; Yu *et al.*, 2004), and more than 25 genes involved in the biosynthesis of aflatoxins via the polyketide pathway have been identified (Norton, 1997; Yu *et al.*, 2004).

At least 16 enzyme-catalysed steps are required to complete the synthesis of aflatoxin B_1 (AFB_1) from norsolorinic acid (NOR), the first stable intermediate. A mutant of *A. parasiticus* has a mutation in the *nor* gene, which codes for norsolorinic acid reductase, and results in a block in aflatoxin biosynthesis. This mutant strain accumulates a coloured compound norsolorinic acid, and is therefore, an excellent tool for the study of aflatoxin pathway inhibitors (Norton, 1997; Sugita-Konishi *et al.,* 2006) because the inhibition can be easily visualised.

Both *A. flavus* and *A. parasiticus* are ubiquitous, and under favourable conditions for infection, these fungi can colonise and grow on a variety of agricultural products. Specifically, aflatoxins have been detected in numerous agricultural commodities, such as cereal grains, whole wheat, rye breads, oil seeds, etc., and principally contaminate maize, cottonseed and peanuts (Bhatnagar and García, 2001; García and Heredia, 2006; Sugita-Konishi *et al.*, 2006).

Any established infection of *A. flavus* can result in rapid accumulation of aflatoxins in the harvested commodity under appropriate temperature and moisture conditions (Hua *et al.*, 1999).

In efforts to control preharvest aflatoxin contamination of commodities, many studies have focused on developing aflatoxin control strategies, including competitive non-aflatoxigenic strains of the fungus *A. flavus*, biological control, crop genetic engineering (Kabak *et al.*, 2006), and regulation of aflatoxin biosynthesis by chemical compounds, such as fungicides, pesticides, inhibitory substances originating from plants, and microbial substances (Mishra and Das, 2003). However, most of these strategies have been shown to be limited in effectiveness.

Consumers demand safe foods without preservatives, or minimally processed commodities (Smith-Palmer *et al.*, 1998). This demand also includes safe feeds which impact on human health. Due to increasing public awareness of the pollutive, residual, carcinogenic, and phytotoxic effects of many synthetic fungicides, the importance of alternative indigenous products to control foodborne mycotoxigenic fungi is increasing (Sánchez *et al.*, 2005). Natural products from plants have been studied as an alternative to control aflatoxin production (Krishnamurthy and Shashikala, 2006).

The genus *Agave* belongs to the *Agavaceae* family that includes more than 300 species. These species are widely distributed, exhibit an exceptional adaptation to drought environments, and provide useful products, including natural fibres, beverages, and potted plants (García-Mendoza, 1995). Previous reports from our laboratory have shown that extracts from certain species of *Agave* such as *A. lecheguilla* Torr., *A. asperrima* Jacobi, *A. striata* Gentri, *A. victoria* Moore, and *A. bracteosa* CAV., were inhibitory to fungal growth, and in some cases to mycotoxin production (Sánchez *et al.*, 2005; Verastegui *et al.*, 1996).

Agave americana is native to the arid and tropical regions of the western hemisphere, particularly Mexico, and Central America. The leaves of this plant are used as fibre and as a folk medicinal herb; they are also used to produce steroidal sapogenins, such as hecogenin, which show anti-inflammatory activity (Jin *et al.*, 2003, 2004). The antifungal activity of this plant has been demonstrated against the growth of *Candida albicans* (Chea *et al.*, 2007), and it has been shown to act as an antisporulant against *Sclerospora graminicola* (Sacc.) Schroet (Deepak *et al.*, 2005).

With the aim of developing alternative aflatoxin control strategies to prevent contamination of agricultural commodities, we investigated the effect of extracts of *A. americana* on the growth, conidiogenesis, and aflatoxin production of *A. parasiticus* SRRC 148 and SRRC 143 strains.

2. Materials and methods

Chemicals

High purity solvents acetone (OmniSolv, EMD Chemicals, Gibbstown, NJ, USA), dichloromethane (OmniSolv, EMD Chemicals), methanol (OmniSolv, EMD Chemicals), and acetonitrile (OmniSolv, EMD Chemicals), sodium chloride (J.T. Baker, Phillipsburg, NJ, USA) and sodium sulfate anhydrous (J.T. Baker) were obtained from VWR International (Goshen Parkway, West Chester, PA, USA). Aflatoxin standards (AFB_1, AFB_2, AFG_1 and AFG_2) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Strains and culture conditions

The fungal strains used in this study were: *A. parasiticus* SRRC 148 and SRRC 143 (SU-1), and *A. parasiticus* SRRC 162, a mutant (*nor*$^-$) that accumulates NOR. These strains were obtained from the USDA, Southern Regional Research Center, New Orleans, LA, USA. *Aspergillus fumigatus* NADC 0073 was used as control strain and was kindly donated by John Thurston, from NADC-USDA, Ames, IA, USA. All strains were maintained on potato dextrose agar (PDA) slants. Inoculum was prepared from PDA cultures incubated for 7 days at 28 °C until they sporulated. Fungal conidia were resuspended in saline solution containing 0.05% Tween 20. The number of conidia (2.5×10^5) in the suspension was adjusted by microscopy using a Neubauer chamber (Sánchez *et al.*, 2005).

Agave americana extracts

Fresh healthy leaves of *A. americana* growing wild on the premises of the city of San Nicolas (NL, Mexico) were collected. Leaves were washed thoroughly under running tap water, and cut into small pieces. To obtain the aqueous extracts, twenty grams of crushed plant materials were ground in 100 ml of sterile distilled water using a blender. The sample was held at 4 °C for 16 h. The suspensions were filtered through Whatman No. 5 filter paper (Whatman, Maidstone, UK). The extracts were then concentrated under reduced pressure on a rotary evaporator at 50 °C and then dried in an oven at 50 °C. The samples were next resuspended in distilled water (4-5 ml) and sterilised by filtration using nitrocellulose membranes (0.45 µm; Millipore, Billerica, MA, USA). The insoluble material was discarded by filtration. Samples were stored at -20 °C until used. An aliquot was used to determine dry weight.

Antifungal assay and conidial count

The susceptibility test was performed in tubes containing 3 ml of Adye and Mateles (A&M) liquid medium (Klich *et al.*, 1993). The tubes were inoculated (1×10^3 conidia/ml, final concentration) using the conidial suspension described

above for each strain. Then, different concentrations (1, 2.5, 5, 10, 20, 40 and 60 mg/ml, final concentration) of *A. americana* extract were added. The tubes were incubated at 28 °C for 7 days, and growth was registered visually every 24 h. The minimal inhibitory concentration was defined as the lowest concentration that completely inhibited any visible fungal growth. The effect of the extract on dried weight and pH of the culture were determined.

To determine the effects that the extract had on conidia production, 0.05% (final concentration) Tween 20 was added to the cultures and vortexed vigorously for 30 s to free conidia. Then, an aliquot was transferred to a Neubauer chamber for conidia enumeration (conidia per ml) as described previously (Sánchez *et al.*, 2005). Triplicate tubes were used for each assay, and results were analysed using the ANOVA test.

Detection of norsolorinic acid

For NOR analysis, 3 ml of A&M liquid medium tubes were inoculated with the *nor*$^-$ mutant *A. parasiticus* SRRC 162 as described above. This strain accumulates NOR, and after incubation (7 days at 28 °C), the culture was filtered, and mycelia were immersed in 5 ml of acetone for ~1 h. The fungal mycelia were then removed, the acetone was evaporated in a fume hood and the residue was then resuspended in 5 ml of 90:10 methanol/1 N NaOH (approximate pH of 10.0; NOR is a pH indicator and is purple at pH 10, which allows for a spectrophotometric assay of NOR at 595 nm). The resulting crude extract was diluted 10- to 50-fold, depending on colour intensity. These data were normalised to a NOR standard, previously purified in our laboratory as previously reported (Keller *et al.*, 1997).

Effect of extracts on toxin formation

A total of 250 µl of a suspension containing 1×10^6 conidia/ml were added to 250 ml Erlenmeyer flasks containing 25 ml of A&M broth in the presence of *A. americana* extracts at differing concentrations (0 and 10 mg/ml, final concentration). At the end of incubation (static for 8 days), separate cultures were used for toxin determination and dried weight. For aflatoxin extraction, 50 ml of acetone and 75 ml of dichloromethane were added to whole A&M cultures. Following the addition of each solvent, the cultures were shake-agitated at 300 rpm for 30 min. Mycelial pellets were removed from the mixture by filtration using Whatman No. 1 paper, and then washed with 25 ml of additional dichloromethane. The filtrate was partitioned in a preparatory funnel into an aqueous phase and a dichloromethane phase that contained most of the aflatoxin and aflatoxin precursors. The aqueous phase was partitioned again with 75 ml of fresh dichloromethane. The two dichloromethane fractions were combined and partitioned against 50 ml of H_2O saturated with NaCl. Residual H_2O was removed from the final dichloromethane solution with 3 g Na_2SO_4, and the solution was evaporated at 40 °C to dryness. The residue was dissolved in 3 ml of aqueous methanol (80:20). Clean-up of aflatoxins took place using an immunoaffinity column (AflaTest, VICAM, Watertown, MA, USA). For HPLC analysis of aflatoxins, samples were separated using a 15-cm LC 18 column (Nova-Pak; Waters, Milford, MA, USA). The mobile phase was water/methanol/acetonitrile (63:26:11) with a flow of 1 ml/min, and detection and quantitation were performed at λ_{em} 365 nm and λ_{ex} 455 nm in a Shimadzu Liquid Chromatograph LC-10vp with a fluorescence detector, RF-10Axl (Shimadzu, Tokyo, Japan). Retention times and concentration of detected toxins were compared with aflatoxin standards (Sánchez *et al.*, 2005). To determine the dried weight of the fungi, the culture was filtered (Whatman No. 1), the fungal mat dried at 50 °C for 72 h and weighed. Supernatants were used for pH determination of the media using a pH meter (Orion mod 310; Thermo Electron Corp., Beverly, MA, USA).

3. Results and discussion

Our results indicate that the *A. americana* extracts did not exhibit inhibitory effects on growth of the studied fungi, but an increase in growth (Figure 1). When different concentrations of aqueous extract of *A. americana* were added to the cultures of *A. parasiticus* SRRC 148, the aflatoxin production per mg of dried weight was reduced significantly ($P<0.05$) in comparison with the control. The reduction was produced by the extracts at concentrations higher than 5-10 mg/ml, where all types of total aflatoxin analysed (AFB_1, AFB_2, AFG_1, and AFG_2) were reduced from 64 to >99% in the whole culture (Table 1). Although a reduction of AFB_1 and AFB_2 was produced, the extracts stimulated fungal growth that resulted in an increased aflatoxin B concentration in the culture; nevertheless the amount was lower when compared with the control (Table1).

Addition of extract to the medium did not substantially change the initial pH of the medium; however after 7 days of growth, the pH decreased in the control to about 2.5; but it increased (above 4.5) as the amount of extract was increased (Figure 2). Aflatoxin synthesis optimally occurs in the pH range of 3.4-5.5 (27); however, addition of extract induced an increase in pH and growth, and still an inhibition of aflatoxin production occurred.

Conidiation assays indicated that varying concentrations of aqueous extracts of *A. americana* had variable effects on conidiogenesis of *Aspergillus* strains. These changes could be visualised directly in the culture (changes in green colour intensity due to conidial formation). Between 1 and 10 mg/ml of extract decreased conidia production; however at higher concentrations, a slight increase in the total amount

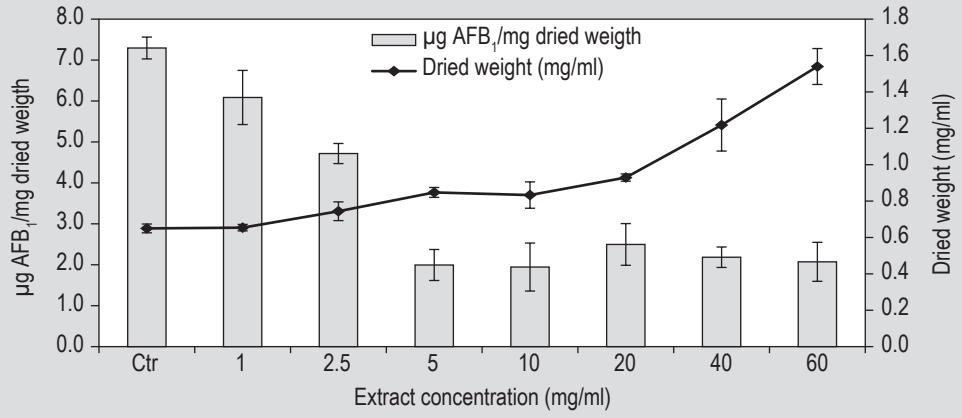

Figure 1. Effect of *Agave americana* on growth and aflatoxin B_1 production of *Aspergillus parasiticus* SRRC 148.

Table 1. Total aflatoxin production in the culture of *Aspergillus parasiticus* following incubation with different concentrations of aqueous extract of *Agave americana*.

Fungal strain		AF production in µg/ml (% of reduction)			
		AFB_1	AFB_2	AFG_1	AFG_2
A. parasiticus SRRC 148	Control	4.7±0.3[a] (0)	5.8±0.2 (0)	1.8±0.1 (0)	1.4±0.05 (0)
	1 mg/ml	4.0±0.3 (16)	5.5±0.1 (4)	1.6±0.09 (14)	1.3±0.05 (8)
	2.5 mg/ml	3.5±0.2 (26)	4.5±0.3 (23)	1.3±0.09 (30)	<0.00008 (>99)
	5 mg/ml	1.7±0.1 (64)	1.8±0.3 (70)	<0.00008 (>99)	<0.00008 (>99)
	10 mg/ml	1.6±0.1 (66)	1.7±0.2 (69)	<0.00008 (>99)	<0.00008 (>99)
	20 mg/ml	2.3±0.2 (51)	2.0±0.2 (64)	1.3±0.1 (27)	<0.00008 (>99)
	40 mg/ml	2.7±0.04 (44)	2.8±0.2 (52)	1.3±0.1 (27)	<0.00008 (>99)
	60 mg/ml	3.2±0.4 (38)	3.0±0.3 (48)	1.4±0.1 (22)	0.9±0.04 (39)

[a] Standard deviation ($P \geq 0.05$)

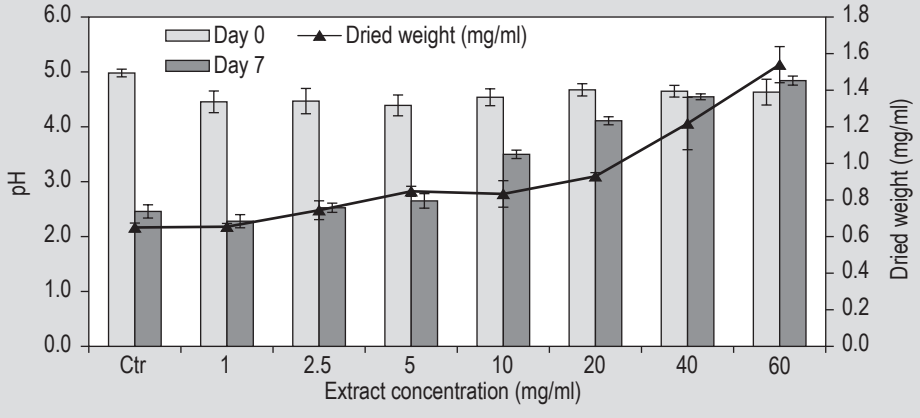

Figure 2. Effect of extracts of *Agave americana* on pH of a culture of *Aspergillus parasiticus* SRRC 148.

of conidia was observed concomitant with the increase in growth (Figure 3). A biphasic pattern in the production of conidia in the culture was observed as a result of adding different amounts of extract. Production of conidia was reduced by lower amounts of the extract, but it increased as higher concentrations of the extract were applied.

A sharp decrease in pigment (NOR) in the cultures was visually observed at extract concentration of 10 mg/ml. Since aqueous extract at 10 mg/ml induced the strongest inhibition of conidiogenesis to *A. parasiticus nor*$^{-}$, this concentration was tested for its effect on NOR production from this mutant. Our results indicate that 10 mg/ml of aqueous extract of *A. americana* caused a significant reduction of 75% (from 0.2±0.02 mg/ml in the control to 0.05±0.01 mg/ml in the culture treated with extract) of the orange-coloured intermediate of the aflatoxin pathway.

The antifungal activity of several other species of *Agave* was recently reported. More specifically, ethanolic, methanolic, and aqueous extracts of *A. asperrima* and *A. striata* were shown to inhibit the growth of *A. flavus* and *A. parasiticus* in culture media and in corn under storage conditions (Sánchez *et al.*, 2005). In the current experiment, the extracts of *A. americana* did not exhibit an inhibitory effect on mycelial growth of *A. parasiticus* at the concentrations examined (0, 1, 2.5, 5, 10, 20, 40, and 60 mg/ml), which may be due to differences in chemical constituents of the agave extracts.

It has been shown that extracts of *A. americana* exhibit remarkable antisporulant effects on *S. graminicola*, the causative agent of pearl millet downy mildew (Deepak *et al.*, 2005). In the current study, extracts of this agave at concentrations between 1 and 10 mg/ml of extract decreased conidia production.

Aflatoxin is synthesised through the polyketide pathway, involving more than 25 genes (Bhatnagar *et al.*, 2002). The first stable intermediate is NOR, and at least 16 consecutive enzyme-catalysed reactions are required to complete the synthesis of aflatoxin from NOR. The *nor*$^{-}$ mutant of *A. parasiticus* has a defective norsolorinic acid reductase, which blocks the aflatoxin biosynthetic pathway, resulting in the accumulation of NOR (Norton, 1997).

Aflatoxin production is related to conidiogenesis in aflatoxigenic *Aspergillus* (Guzmán and Ruíz-Herrera, 2002). In the Aspergilli, one of the first genetic insights linking sporulation with mycotoxin production was that both are regulated by members of a G protein signalling pathway (Brodhagen and Keller, 2006; McDonald *et al.*, 2004). Intensive subsequent genetic and biochemical exploration of this pathway has begun to reveal the signalling circuitry connecting aflatoxin / sterigmatocystin production and sporulation in *Aspergillus*. Mutations in G protein signalling pathway genes in *A. parasiticus* induced a decrease in conidiation, and a loss of NOR (aflatoxin precursor), while these mutations in *A. flavus* induced decreased conidiation and repressed production of aflatoxin and cyclopiazonic acid (Bhatnagar *et al.*, 2006). Here, the concentrations of extract of *A. americana* that decreased conidiogenesis also strongly inhibited aflatoxin production. These results are supported by reports indicating that extracts of other agaves such as *A. asperrima* and *A. striata* were able to inhibit production of aflatoxin and cyclopiazonic acid (Sánchez *et al.*, 2005).

At the present time, it is not possible to accurately identify the mechanism that affects conidiogenesis and aflatoxin production. The effect may be at the level of the synthesis of the polyketide precursor, since the synthesis of the first known precursor, NOR, was negatively affected (Bennett and Klich, 2003).

A. americana is native to the arid and tropical regions and it is widely distributed in several parts of the world. It is easy to grow and its availability makes it feasible to use as an economical resource. Furthermore, the aqueous extract of

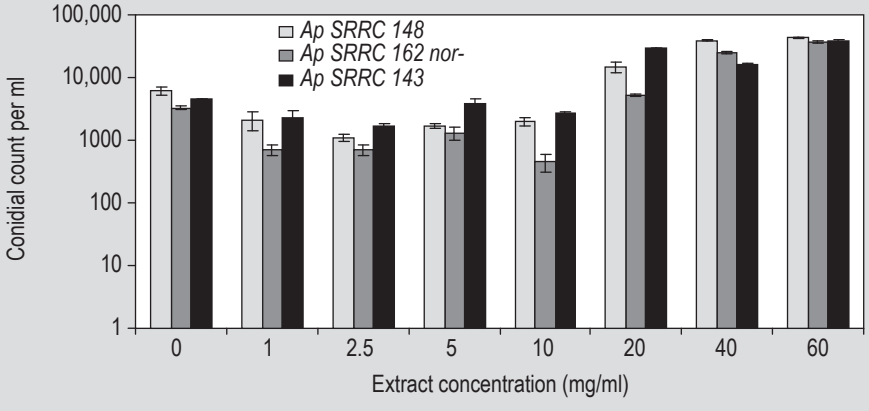

Figure 3. Conidial production of *Aspergillus parasiticus* (*Ap*) strains in the presence of aqueous extract of *Agave americana*.

this plant is easy to prepare and handle. The high prevalence of aflatoxin contamination of food and feeds in the world requires the use of inexpensive, natural alternatives that help to control this problem in different environments. The results of the present work indicate that extracts of *A. americana* may be promising safe alternatives to harmful fungicides for controlling aflatoxin contamination in foods and feeds. Toxicity studies, and experiments to determine the specific compounds responsible for the antimicrobial activity of the extract, are in progress.

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